1 OBJECTIVE

The objective of this study was to validate the residue analytical method for the determination of IKF-5411 and its metabolite 4HP in soil.

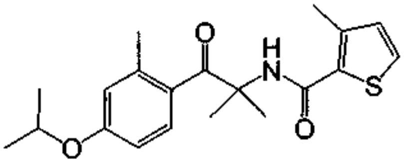
2 CONDUCT OF STUDY

The study was conducted at Ishihara Sangyo Kaisha, Ltd., Central Research Institute, Safety Science Research Laboratory, Environmental Sciences Group, 3-1, 2-Chome, Nishi-shibukawa Kusatsu-shi, Shiga-ken, 525-0025 Japan. The experimental start and termination dates were January 5, 2010 and January 25, 2010, respectively.

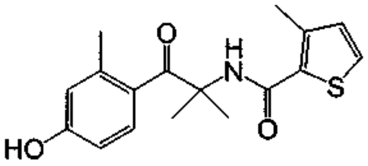
3 MATERIALS AND METHODS

3.1 TEST ITEMS

3.1.2 IKF-5411

Product Name:	IKF-5411
Chemical Name:	N-[1,1-dimethyl-2-[2-methyl-4-(1-methylethoxy)phenyl]-2-oxoethyl]-3-methyl-2-thiophenecarboxamide (CA)
Lot No.:	080115
Purity:	99.1 %
Molecular Weight:	359.48
Chemical Structure:	

3.1.2 4HP

Product Name:	4HP
Chemical Name:	N-[1,1-dimethyl-2-(4-hydroxy-2-methylphenyl)-2-oxoethyl]-3-methyl-2-thiophenecarboxamide (IUPAC)
Lot No.:	20091026
Purity:	98.4 %
Molecular Weight:	317.4
Chemical Structure:	

3.2 TEST SOILS

Two soils received from Huntingdon Life Sciences (HLS) in October 2009 were used in this study. Soil properties were presented in Table 1.

3.3 REAGENTS AND APPARATUS

All reagents were of analytical, HPLC, or LC/MS/MS grade.

REAGENTS & APPARATUS	ARTICLE No.	SUPPLIER
Purified water		Wako Pure Chemical
Acetonitrile		Wako Pure Chemical
Methanol		Nacalai Tesque
Hydrochloric acid (6 mol/L)		Nacalai Tesque
Acetic acid		Wako Pure Chemical
Celight	545 RVS	Nacalai Tesque
Erlenmeyer flask with ground glass stopper	200 mL	Iwaki
Volumetric flasks	various sizes	Iwaki
Volumetric pipettes	various sizes	Iwaki
Mesureing flasks	various sizes	Iwaki
Glass funnel		Iwaki
µL-Pipettes	1000 µL	Gilson
Conical tube	15 mL PP	BD Bioscience
SPE Cartridge	OASIS HLB VAC RC (60mg)	Waters
SPE manifold		Waters
HPLC vaial	LC/MS certificated	Waters
Analytical balance	AUW220D	Shimadzu
Laboratory balance	UX4200S	Shimadzu
Reciprocal Shaker	SR-2	TAIYO
Oven	MODEL 2-2132	ISUZU SEISAKUSHO

3.4 STANDARD SOLUTIONS

3.4.1 Stock solutions

10.0 mg of IKF-5411 and 4HP was weighed into 100 mL-Volumetric flask, respectively. Acetonitrile was added to make a stock standard solution with a concentration of 100 µg/mL.

3.4.2 Fortification solutions

The stock solution of 100 µg/mL IKF-5411 and 4HP were mixed and diluted using acetonitrile to obtain a mixed fortification solution with a concentration of 40 µg/mL IKF-5411 and 4HP.

The stock solution of 100 µg/mL IKF-5411 and 4HP were mixed and diluted using acetonitrile to obtain a mixed fortification solution with a concentration of 10 µg/mL IKF-5411 and 4 HP.

The mixed standard solution of 1 µg/mL IKF-5411 and 4HP was diluted using acetonitrile:water (80:20, v/v) to obtain a mixed fortification solution with a concentration of 100 ng/mL IKF-5411 and 4HP.

3.4.3 Calibration solutions

A mixed calibration solutions, over the concentration range 0.01 to 2 ng/mL IKF-5411 and 4HP, were prepared by serial dilution of the mixed fortification solutions IKF-5411 and 4HP in acetonitrile:water (80:20, v/v).

3.5 FORTIFICATION

To demonstrate the validity of the method used, untreated soils were fortified with different amounts of the IKF-5411 and 4HP.

Fortification levels for soil:

0.01 mg/kg:	2 mL of the mixed fortification solution (100 ng/mL) was added to 20 g (dry mass) soil.
0.5 mg/kg:	1 mL of the mixed fortification solution (10 µg/mL) was added to 20 g (dry mass) soil.
2 mg/kg:	1 mL of the mixed fortification solution (40 µg/mL) was added to 20 g (dry mass) soil.

3.6 ANALYTICAL METHOD

3.6.1 Extraction

20 g (dry mass) of the untreated soil sample was weighed into a 200 mL Erlenmeyer flask with ground glass stopper. 100 mL of acetonitrile:water (80:20, v/v) and 1 mL of 6 mol/L hydrochloric acid were added to the soil sample. The sample was shaken for 30 minutes using a reciprocal shaker. The mixture was filtered through a Celite 545. The filter cake was washed with 80 mL of acetonitrile:water (80:20, v/v). The filtrate and washings were combined and then filled up to 200 mL with acetonitrile:water (80:20, v/v).

3.6.2 Sample clean up on SPE

A SPE cartridge (OASIS HLB VAC RC, 60 mg) was placed onto a SPE vacuum manifold and conditioned using methanol (5 mL) followed by water (5 mL). 1 mL of the extract and 10 mL of water were mixed and transferred into the SPE cartridge. The aqueous sample solution was sucked through the column followed by 4 mL of acetonitrile:water (20:80, v/v). All eluates were discarded. IKF-5411 and 4HP were eluted with 10 mL of acetonitrile:water (80:20, v/v). The eluate was collected and then filled up to 50 mL with acetonitrile:water (80:20, v/v).

3.6.3 Preparation for the LC/MS/MS analysis

Aliquot of the sample was sealed in HPLC vial for injection. The sample fortified with 2 mg/kg IKF-5411 and 4HP were diluted ten times with acetonitrile:water (80:20, v/v). The concentration of IKF-5411 and 4HP in sample solution was determined by LC/MS/MS analysis

3.6.4 Quantitation

Quantitation of the IKF-5411 and 4HP concentration was performed by LC/MS/MS using the external standard method. The calibration standards at six concentrations (0.01, 0.02, 0.04, 0.2, 0.4, 2 ng/mL) were used for construction of a calibration curve. The calibration curve was constructed by plotting the peak areas against the concentration of calibration standards. From the calibration curve, the concentration of IKF-5411 and 4HP in the injected solution was determined and the residue of IKF-5411 and 4HP in soil sample was calculated.

3.7 LC/MS/MS CONDITIONS

3.7.1 Part A:HPLC

Instrument:	ACQUITY UPLC System (Waters)
Column:	BEH C_{18} 2.1×50 mm, 1.7 μm (Waters)
Guard column:	VanGuard BEH C_{18} 2.1×5 mm, 1.7 μm (Waters)
Column temp.:	40 °C
Mobile phase:	Acetonitrile:Water:Acetic acid (70:30:0.1, v/v/v)
Flow rate:	0.5 mL/min
Injection volume:	4 μL

3.7.2 Part B:MS/MS

Instrument:	API4000QTRAP (Applied Biosystems)
Ionization mode:	ESI
Scan mode:	MRM
Mass resolution	Q1;unit, Q3;low
Heater gas temp.:	500 °C
Ion voltage:	5000 V
Gas flow settings:	Gas1;60, Gas2;90, CUR;15, CAD;6

Primary method

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
IKF-5411	Pos. $[M+H]^+$	360.2	209.9	15	61	10	20
4HP	Pos. $[M+H]^+$	318.1	125.0	52	61	10	20

Confirmatory method

Analyte	Ion Polarity	Precursor Ion (m/z)	Product Ion (m/z)	CE	DP	EP	CXP
IKF-5411	Pos. $[M+H]^+$	360.2	124.9	39	61	10	20
4HP	Pos. $[M+H]^+$	318.1	210.2	15	61	10	18

Mass spectra of IKF-5411 and 4HP were presented in Figure 1 and 2, respectively.

3.8 CALCULATION

The residue of IKF-5411 and 4HP in soil was calculated according to equation 1.

$$R = \frac{X \times V_F \times D}{W \times 1000} \quad (1)$$

Where

R = Residue of IKF-5411 and 4HP in soil sample [mg/kg]

X = Concentration of injected solution [ng/mL]

V_F = Final Volume [50 mL]

D = Dilution Factor [if applicable]

W = Aliquot of sample [0.1 g]

1000 = Conversion factor from ng to µg

The recovery of IKF-5411 and 4HP in soil was calculated according to equation 2.

$$Rec = \frac{R \times 100}{F} \quad (2)$$

Where

Rec = Recovery of IKF-5411 and 4HP [%]

R = Residue of IKF-5411 and 4HP in soil sample [mg/kg]

F = Fortification level [mg/kg]